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(57) Abstract

The invention relates to the use of nucleic acid sequences coding for polygalacturonase in the control of dehiscence in plants. Plants transformed with such nucleic acid sequences are also disclosed.

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CONTROL OF POD DEHISCENCE

This invention relates generally to the control of pod dehiscence or shatter.

Abscission is the process that causes the shedding of a range of plant parts, including leaves, flowers and fruit. The process occurs at precise sites and involves coordinated cell wall breakdown. Associated with cell separation is an increase in the activity of several hydrolytic enzymes including β-1,4-glucanase (cellulase, EC 3.1.2.4) and polygalacturonase (PG EC 3.2.1.15).

The process of pod dehiscence, or shatter as it is commonly termed, in oilseed rape (*Brassica napus*) and other crops shares a number of features with abscission. Degradation and separation of cell walls occurs along a discrete layer of cells, termed the dehiscence zone, and a localised increase in the activity of cellulase has been reported prior to the onset of dehiscence (Meakin and Roberts *J. Exp. Bot.* 41(229) 995-1002 (1990) and *J. Exp. Bot.* 41(229) 1003-1011 (1990)). This process is agronomically important because it may result in the premature shedding of seed before the crop can be harvested. Adverse weather conditions can exacerbate the process resulting in a greater than 50% loss of seed. This loss of seed not only has a dramatic effect on yield but also results in the emergence of the crop as a weed in the subsequent growing season.

Attempts to solve this problem over the last 20 years have focused on the breeding of shatter-resistant varieties. The most commonly used method is by trying to introduce germplasm from related species by interspecific hybridisation. Related species such as *B. nigra, B. juncea* and *B. campestris* have been used for this purpose but resulting plants from these crosses are frequently sterile and lose favourable characteristics which have to be regained by back crossing. This is both

tim consuming and laborious. The interspecific hybridisation strategy also has to cope with transferring two or more genes which are recessive in action into each of the breeding lines. Indeed, even within *B. campestris*, different genetic backgrounds have revealed different numbers of genes to be important in shatter resistance. This has necessitated breeders performing test crosses at each generation during the attempt to produce elite material. These difficulties have been compounded by the fact that shattering is a difficult and time-consuming trait to assess in the field. All these factors may account for the fact that the conventional breeding approach has made no progress over the last twenty years.

Other methods employed to try and alleviate the problem include chemicals, in the form of desiccants and pod sealants. The most widely used method to try and prevent seed loss is the mechanical technique of swathing in order to get uniform desiccation of the crop and reduce shattering by wind which occurs in the upright crop.

This invention takes a completely different approach to solve the problem of dehiscence : it involves the use of recombinant DNA technology. In 1988, when plant biotechnology had reached an age of some considerable sophistication, Roberts and Taylor speculated:

> By regulating cell separation at abscission sites, it may be possible...to also influence related processes such as pod dehiscence. (*Proceedings of the Symposium on the Physiology of Fruit Drop, Ripening, Storage and Post-Harvest Processing of Fruits*, Turin, 3-4 October 1988, pp 24-33).

However, without any indication of which genes may be involved in such processes, this exhortation did little to enable the art to address the problem at the cell or gen tic level.

WO94/23043 discloses nucleic acid sequences ncoding proteins

involved in plant abscission or dehisc nce. However this disclosure does not include any discussion of polygalacturonase's role in abscission or dehiscence.

Meakin and Roberts (*supra*) reported that there was no correlation between the timing of dehiscence and the activity of the pectin degrading enzyme, polygalacturonase (PG). Although they stated that it was not possible to discount a role for PG in pod shatter, their work did not provide any evidence that activity of the enzyme was related to timing of dehiscence.

It has now been discovered that, in fact, PG is implicated in dehiscence and that manipulation of this enzyme's activity can influence the timing of dehiscence. The gene coding for PG has a pattern of expression which is, spatially and/or temporally, specific or at least preferential for tissue involved in dehiscence. The invention relates to the exploitation of the gene and related DNA sequences (including regulatory sequences) in the manipulation of pod dehiscence including its reduction or prevention in particular.

According to a first aspect of the present invention, there is provided the use of a recombinant or isolated nucleic acid sequence which encodes the enzyme polygalacturonase in the control of dehiscence.

The invention has application to all crops which have a dehiscence zone and may therefore lose seed pre-harvest because of cell separation events. An economically important plant genus to which the invention can be applied is that of *Brassica*, eg the important crop *Brassica napus*.

In patticular, the polygalacturonase will be prefer ntially or specifically expressed in the d hisc nce zone of pericarp tissu . In n embodiment, th pericarp tissue is from the genus *Brassica*, pr ferably *Brassica napus*. Suitably, the nucleic acid sequenc will be a DNA sequence.

Recombinant or isolated nucleic acid sequences which can be used in this invention are illustrated by the nucleic acid sequence of Figure 1, which encodes the amino acid sequence shown in that figure. All other nucleic acid sequences which, by virtue of the degeneracy of the genetic code, also code for the given amino acid sequence are also useful for the practice of the present invention. Nucleic acid sequences which are substantially homologous to nucleic acid sequences encoding the amino acid sequence shown in Figure 1 can also be employed.

"Substantial homology" may be assessed either at the nucleic acid level or at the amino acid level. At the nucleic acid level, sequences having substantial homology may be regarded as those which hybridise to the nucleic acid sequences shown in Figures 3 and 8 under stringent conditions (for example at 35 to 65°C in a salt solution of about 0.9M). At the amino acid level, a protein sequence may be regarded as substantially homologous to another protein sequence if a significant number of the constituent amino acids exhibit homology. At least 40%, 50%, 60%, 70%, 80%, 90%, 95% or even 99%, in increasing order of preference, of the amino acids may be homologous.

The skilled man will also appreciate that modified proteins may also be employed. "Protein engineering" techniques are now well established in the art and it is within the scope of the present invention to utilise polygalacturonase enzymes which have been so "engineered" to modify their properties. For instance, their specificity or kinetics may be altered in some way in order to be more effectice. The essential feature which they must retain of course is activity as a polygalacturonase enzyme.

Preferably, the nucleic acid used in the invention will include a promoter or other regulatory sequence which naturally controls expression of polygalacturonase. Since it has been found that PG is expressed during dehiscence, such promoters are themselves useful in controlling this process. The skilled man will appreciate that it may not be necessary to

utilis the whole promoter. Only essential r gulatory lements may be us d and in fact such elements can be used to construct chimeric sequences or promoters. The essential requirement is of course to retain the tissue and/or temporal specificity.

In a second aspect, the invention provides the use of the enzyme polygalacturonase in the control of dehiscence.

As described above the promoters or other regulatory sequences (including synthetic or chimeric promoters) which are capable of driving expression of polygalacturonase in the dehiscence zone are also useful in controlling dehiscence. Thus, in a third aspect, the present invention provides a nucleic acid sequence which is a promoter or other regulatory sequence which naturally controls expression of polygalacturonase.

In a fourth aspect, the present invention provides the use of such promoters or other regulatory sequences in the control of dehiscence.

While promoters as described above may drive DNA encoding an enzyme, they may alternatively drive DNA whose transcription product is itself deleterious. Examples of such transcription products include sense constructs (which may act by co-suppression), antisense RNA and ribozymes.

As far as antisense nucleic acid is concerned, introducing the coding region of a gene in the reverse orientation to that found in nature can result in the down-regulation of the gene and hence the production of less or none of the gene product. The RNA transcribed from antisense DNA is capable of binding to, and destroying the function of, a sense RNA of the sequence normally found in the cell, thereby disrupting function. Examples of suitable antisense DNAs are the antis nse DNAs of the sequence shown in Figure 1. Since this sequenc is normally expr ssed in th d hiscence zone, antisense sequenc s to it may be expected to

disrupt normal dehiscence.

Ribozymes are RNA "enzymes" capable of highly specific cleavage against a given target sequence (Haseloff and Gerlach, *Nature* 334 585-591 (1988)).

Promoters useful as described above may be located in cDNA or genomic libraries using, for example, probe sequences taken from the nucleic acid sequence of Figure 1.

Nucleic acid useful in the invention includes that which, when introduced into a plant, prevents or otherwise interferes with normal dehiscence by interfering with the normal expression of polygalacturonase. Of course, dehiscence-specific promoters, as discussed above, may be useful in this feature of the invention. However, there is a broader dimension which must be considered: antisense DNA or ribozyme-encoding DNA specific for polygalacturonase need not be driven by dehiscence-specific promoters. Instead, they could be driven by constitutive or other promoters (such as for example the CaMV 35S, rubisco or plastocyanin promoter). If the sense gene is only expressed in the pod, there will with an antisense approach be no pleiotropic effects on plant development, and only the development of the dehiscence zone will be disrupted. A ribozyme gene expressed throughout the plant will not result in a translated protein product, and so may require less metabolic energy than the synthesis of a gene product throughout most of the plant.

Antisense technology and ribozyme technologies have already found application in other areas of plant molecular biology. For example, antisense technology has been used to control tomato fruit ripening. Rib zyme technology has been used to control viral infection of melons.

While DNA or RNA in accordance with this f ature of the inventi n generally interferes with th proper expression of polygalacturonase genes

during pod development, in preferred embodiments expression is substantially prevented.

In preferred embodiments of the invention, 3'-transcription regulation signals, including a polyadenylation signal, may be provided as part of the nucleic acid sequences. Preferred 3'-transcription regulation signals may be derived from the cauliflower mosaic virus 35S gene. It should be recognised that other 3'-transcription regulation signals could also be used.

The nucleic acid used in the invention may be in the form of a vector. The vector may for example be a plasmid, cosmid or phage. Vectors will frequently include one or more selectable markers to enable selection of cells transfected (or transformed: the terms are used interchangeably in this specification) with them and, preferably, to enable selection of cells harbouring vectors incorporating heterologous DNA. Appropriate start and stop signals will generally be present. Additionally, if the vector is intended for expression, sufficient regulatory sequences to drive expression will be present; however, nucleic acid for use in the invention will generally be expressed in plant cells, and so microbial host expression would not be among the primary objectives of the invention, although it is not ruled out. Vectors not including regulatory sequences are useful as cloning vectors.

Cloning vectors can be introduced into *E. coli* or another suitable host which facilitate their manipulation. According to another aspect of the invention, there is therefore provided a host cell transfected or transformed with nucleic acid as described above.

Nucleic acid for use in the invention can be prepared by any convenient method involving coupling together successiv nucleotides, and/or ligating oligo- and/or poly-nucleotides, including *in vitro* processes, but recombinant DNA techn logy forms th method of choice.

Ultimately, nucleic acid for use in the invention will where appropriate be introduced into plant cells, by any suitable means.

Preferably, nucleic acid is transformed into plant cells using a disarmed Ti-plasmid vector and carried by *Agrobacterium* by procedures known in the art, for example as described in EP-A-0116718 and EP-A-0270822. Alternatively, the foreign nucleic acid could be introduced directly into plant cells using an electrical discharge apparatus. This method is preferred where *Agrobacterium* is ineffective, for example where the recipient plant is monocotyledonous. Any other method that provides for the stable incorporation of the nucleic acid within the nuclear DNA of any plant cell of any species would also be suitable. This includes species of plant which are not currently capable of genetic transformation.

Preferably nucleic acid for use in the invention also contains a second chimeric gene (a "marker" gene) that enables a transformed plant containing the foreign nucleic acid to be easily distinguished from other plants that do not contain the foreign nucleic acid. Examples of such a marker gene include antibiotic resistance (Herrera-Estrella *et al., EMBO J.* 2(6) 987-95 (1983) and Herrera-Estrella *et al., Nature* 303 209-13 (1983)), herbicide resistance (EP-A-0242246) and glucuronidase (GUS) expression (EP-A-0344029). Expression of the marker gene is preferably controlled by a second promoter which allows expression in cells other than the tapetum, thus allowing selection of cells or tissue containing the marker at any stage of regeneration of the plant. The preferred second promoter is derived from the gene which encodes the 35S subunit of Cauliflower Mosaic Virus (CaMV) coat protein. However any other suitable second promoter could be used.

A whole plant can be regenerated from a single transformed plant cell, and the invention therefore provides transgenic plants (or parts of them, such as propagating material) including nucleic acid as defined herein. The regeneration can proceed by known methods.

Thus, in further aspects, the present invention provid s:

(i) a transgenic plant which includes a nucleic acid sequence as defined herein;

(ii) an oilseed rape plant which is shatter resistant;

(iii) a plant cell which includes a nucleic acid sequence as defined herein;

(iv) propagating material derived from transgenic plants of the invention;

(v) seeds derived from transgenic plants of the invention;and

(vi) a method of regulating dehiscence, which comprises the step of transforming or transfecting propagating material from a plant with a nucleic acid sequence as defined herein.

Preferred features of each aspect of the invention are as for each other aspect *mutatis mutandis*.

The invention will now be illustrated by the following Examples. The Examples refer to the accompanying drawings, in which:

FIGURE 1: shows the nucleic acid and corresponding amino acid sequence of SAC66;

FIGURE 2: shows the sequence of the 164bp PCR fragment of SAC66;

FIGURE 3: shows the northern blot analysis of SAC66;

FIGURE 4: shows the results of hybridisations using the sequence of Figure 1 of SAC66 to southern blots of *B.napus* genomic DNA digested with *Eco*RI(E), *Hind*III(H) and *Bam*HI(B);

FIGURE 5: shows the results of RT-PCR analysis of RNA extracted from the dehiscence zone (A) and non-zone tissues (B);

FIGURE 6: shows the results of RT-PCR analysis of RNA extracted from embryo (E) and funiculus tissues (F) as well as combined DZ and NON-Z regions of of the pod wall valves from *B.napus*;

FIGURE 7: shows the results of the Ribonuclease protection assay described in example 3; and

FIGURE 8: shows the construction of expression cassettes as described in example 5.

EXAMPLE 1 - Cloning of pSAC66

Plant Material

Seeds of *B. napus* cv Rafal were grown as described by Meakin and Roberts, (*J. Exp. Bot.* 41(229) 995-1002 (1990)) with the following modifications. Single seedlings were potted into 10 cm pots containing Levington M2 compost, and after vernalisation for 6 weeks, were re-potted into 21 cm pots. Infection by powdery mildew or aphids was controlled by the application of Safers fungicide. At anthesis, tags were applied daily to record flower opening. This procedure facilitated accurate age determination of each pod. Pods were harvested at various days after anthesis (DAA). The dehiscence zone was excised from the non-zone material and seed using a scalpel blade using the method of Meakin and Roberts (*J. Exp. Bot.* 41: 1003-1011 (1990)) and immediat ly frozen in liquid N_2 prior to storage at -70°C.

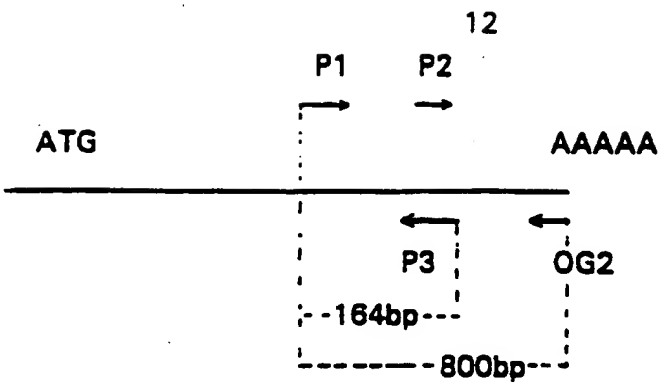
RNA Isolation

All chemicals were molecular biology grade and bought from either Sigma Chemical Ltd (Dorset, UK), or ICN Biomedicals. Total RNA was extracted using the polysomal extraction method of Christoffersen and Laties, *Proc. Natl. Acad. Sci.* 79 4060-4063 (1982), with the following alterations. The plant material was ground to a powder in liquid N_2 and then in 10 volumes of extraction buffer (200 mM Tris-acetate [pH 8.2], 100 mM magnesium acetate, 20 mM potassium acetate, 20 mM EDTA, 5% w/v sucrose, after sterilisation 2-mercaptoethanol was added to 15 mM and cycloheximide added to a final concentration of 0.1 mg ml^{-1}). The supernatant was then layered over 8 ml 1M sucrose made with extraction buffer and centrifuged in a KONTRON™ (Switzerland) TFT 70.38 rotor at 45,000 rpm (150,000 g) for 2 hr at 2°C in a Kontron CENTRIKON™ T-1065 ultra-centrifuge. Pellets were then resuspended in 500 µl 0.1M sodium acetate, 0.1% SDS, pH 6.0 and phenol/chloroform (1:1 v/v) extracted and the total RNA precipitated. mRNA was isolated from the total population of nucleic acids extracted from the dehiscence zone and non-zone tissue at 40, 45 and 50 DAA pods, using the Poly(A) quick mRNA purification kit (Stratagene, cambridge UK), and was used to make 1st strand cDNA using reverse transcriptase.

PCR with degenerate primers

Degenerate primers were designed by multiple sequence alignments of known fruit-specific and pollen-specific polygalacturonase (PG) mRNA sequences. Two primers were synthesized in the 5' to 3' direction (P1 and P2) to prime with a polyT primer (OG2) and one in the 3' to 5' direction (P3) for a nested PCR approach with P1.

Sequences of each primer and the relative positions of P1, P2 and P3 on a PG mRNA sequence are shown below.



Degenerate primers were designed on PG consensus amino acids. Restriction enzyme sites were included in each primer.

P1: (EcoRI) PNTDG

P2: (EcoRI) CGPGHG

P3: GPGHG (BamHI)

OG2: 5' GAGAGAGGATCCTCGAG....T(x17) 3'

Each PCR reaction contained 1 X PCR buffer, 1.5U Taq polymerase, 2 μg degenerate primer, 200 ng non-degenerate primer and 2 ng first strand cDNA in a total volume of 20 μl was overlaid with 18 μl mineral oil. PCR buffer (1X) contained 45 mM tris-HCl pH 8.8, 11 mM ammonium sulphate, 4.5 mM magnesium chloride, β-mercaptoethanol, 4.5 mM EDTA pH8, 10 mM of each dNTP and 0.1 mg ml BSA.

PCR cycles were as follows: 20 PCR cycles included a denaturation temperature of 94°C for 1 min, primer annealing temperature of 45°C for 2 min and extension temperature of 72°C for 2 min. During the following 5 cycles, the Taq polymerase extension time was increased to 2.5 min and in the final cycles for 3 min. The reactions were carried out on a Techne Thermocycler model PHC-3 using a ramp rate of 1°C/sec.

A sample of th PCR r action (10 μl) was run on a 2% agar s g l and DNA of the predicted siz eluted using either a "gelase" kit (nbl g ne sciences Northumberland UK) for pr ducts less than 500bp r a "gen clean" kit (Bio. c/o Statech, Cambs, UK) for products greater than

500bp. The final pellet was resuspended in 20 μl, and 2 μl were used in subsequent PCR reactions.

cDNA Library Screening

Three plates each containing approximately 40,000 recombinant plaques from a dehiscence-related cDNA library generated by Coupe *et al, Plant Mol. Biol.* (1993), were screened with a radio-labelled 164bp (shown in figure 2) fragment derived from the nested PCR approach described above using *in situ* plaque hybridisation. Duplicate plaque lifts were obtained using HYBOND™ N^+ membranes (Amersham, Aylesbury, UK) and were then treated and hybridised according to manufacturers' instructions at 65°C in the presence of formamide (50%). Membranes were washed at 65°C in 2 X sodium chloride, sodium phosphate, EDTA (SSPE) 0.1% SDS. Hybridising plaques were re-screened at densities of 50-100 plaques/plate. Individual plaques hybridising in a 2nd round were cored from the plate, rescued into SM buffer with chloroform, and phage inserts isolated by PCR from boiled aliquots of each coring. Inserts of varying sizes were visualised on a 1XTAE gel. A Southern blot of the gel probed with the 164bp fragment showed strong hybridisation to a phage insert of 1.7kb. Plasmids were isolated from appropriate phage stocks using the *in vivo* excision procedure (Short *et al, Nucl. Acids Res.*, **16**: 7583-7600 (1988). The cDNA containing insert of 1.7kb was designated pASC66.

Northern Blot Analysis of RNA

10μg total RNA isolated from various parts of the oilseed rape plant were separated on a (1% agarose/3% formaldehyde/10mM Na_2HPO_4 pH 6.5) denaturing gel. The RNA was transferred onto a nylon membrane (GeneScreen, NEN-Du Pont, UK) using capillary transfer. In accordance with the membrane manufacturers' instructions, a riboprobe was transcribed from pSAC66 using the method outlined in the Promega protoc ls and applications guide, 2nd edition (1991) p59. Unincorporated radionucleotid s were removed with a Biogel P-60 column. The blot was washed at 65°C in 0.1 x SSPE, 0.1% SDS and exposed to KODAK™ X-

AR5 film with intensifying screens at -70°C.

DNA Sequencing

Supercoiled plasmid DNA from pSAC66 was isolated by the alkaline-lysis method of Sambrook *et al, Molecular Cloning: A Laboratory Manual* New York, Cold Spring Harbour Laboratory Press (1989) phenol/chloroform extracted and treated with RNase A (Sigma UK) and precipitated with PEG. Sequence data were obtained using an Applied Biosystems automated sequencer employing Taq dyedeoxy terminators. Initial sequence of SAC66 was derived using pBluescript bacteriophage primer T3 and T7 and subsequently using internal primers.

Isolation of B. napus fragment encoding PG by PCR with degenerate primers

First strand cDNA was synthesized from *B. napus* mRNA isolated from the DZ of pods harvested at 40, 45 and 50 DAA. *B. napus* cDNA was used in PCR reactions with degenerate primers whose design was based on multiple sequence alignments of fruit-specific and pollen-specific PGs. Two primers were synthesized in the 5' to 3' direction (P1 and P2) to prime with a polyT primer (OG2) and one in the 3' to 5' directoin (P3) for a nested PCR approach with P1.

First round PCR products from *B. napus* cDNA were visible as smears in the appropriate size range for all primer combinations (P1 + OG2, P2 + OG2 and P1 + P3). Gel fragments were excised in the expected product size of each primer combination and the DNA eluted for second round PCR reactions. A band of less than 200bp was visible in the P1 + P3 primed reactions from first round PCR products P1 + OG2, i.e. nested PCR. This 164bp fragment was cloned and the sequence (Figure 2) showed significant homology to other PGs within the database.

Homologous screening of the DZ cDNA library for a clone encoding PG

using a PCR product

Three plates each containing approximately 40,000 clones from a dehiscence zone cDNA library were screened with the 164bp fragment derived from the nested PCR approach. Distinct hybridisation plaques were taken through a second round of screening and phage inserts amplified by PCR from boiled aliquots of corings. The largest insert was designated as SAC66 and the phage containing this insert were *in vivo* excised and plasmid DNA prepared for sequencing.

pSAC66 sequence and amino acid analysis

Both strands of the cDNA were sequenced and the result is shown in Figure 1. When sequenced, the cDNA was 1657bp in length. The largest open reading frame (ORF) was 1299 nucleotides in length, beginning at position 145. The deduced protein sequence of 433 amino acids has a calculated molecular mass of approximately 45 kDa. At the end of the nucleotide sequence there was a large poly(A) tail that encompassed 18(A) nucleotides. The sequence shows close amino acid homology to other polygalacturonases including that isolated from tomato and kiwifruit.

Spatial and temporal expression of SAC66

Northern analysis revealed that the 1657bp insert from pSAC66 hybridised to a mRNA of approximately 1.7kb that increased in expression specifically in the dehiscence zone (DZ) tissue excised from pods 45 or 50 DAA. The process of dehiscence is visible to the naked eye at about 50 DAA. No expression of this mRNA could be seen in non-zone pod tissues.

Genomic Southern analysis of SAC66

The 1657bp insert of pSAC66 was used as a probe for hybridisation to Souther blots of *B. napus* genomic DNA digested with *Eco*R1, *Hind*III and *Bam*H1 (Figur 4). Th probe hybridised to sev ral fragments ranging in

size from 12.2 to 0.6kb. The data suggest that, in this respect, PG is a member of a multi-gene family.

Example 2-Genomic DNA Isolation and Characterisation

DNA was isolated, using a miniprep procedure using a modified form of the extraction buffer described by Clarke *et al*, *Genome* **32**: 334-339 (1989). Young expanding oilseed rape seedlings were homogenised in a 3.8:0.6:0.5 mixture of the following; TNE buffer (0.05M Tris.HCl pH 7.5, 0.2 M EDTA: 0.1 M NaCl): 5% SDS: 1 mg ml^{-1} proteinase K; to this solution was added sodium diethyldithiocarbamate and sodium bisulphite to 0.4% (w/v) just before use. The samples were then incubated for 1 hr at 37°C and debris removed by centrifugation in a microfuge at 11,600g for 5 min. The eluate was then extracted with equal volumes of phenol/chloroform (1:1 v/v) and then chloroform alone. Nucleic acids were then precipitated by the addition of 2.5 vols 95% ethanol containing 5% (v/v) 2M Na acetate, pH 5.5. The sample was then mixed and immediately centrifuged at 11,600g for 5 min. The resulting pellet was resuspended in 300 µl TE, 10µl of RNaseA (10 mg ml^{-1}) added, and then incubated at 37°C for 15 mins before 300 µl CTAB buffer (0.2M Tris.HCl pH 7.5, 0.05 M EDTA, 2 M NaCl and 2% w/v CTAB) was added before a further incubation at 60°C for 15 mins. Following re-extraction with an equal volume of chloroform, the DNA was precipitated with an equal volume of isopropanol at -20°C. Subsequent digestions by restriction endonucleases were carried out as detailed in Stacey and Isaac, Restriction enzyme digestion, gel electrophoresis and vacuum blotting of DNA to nylon membranes (1993). The DNA was then separated in 1 X TBE, 0.8% agarose and transferred to GENESCREEN+™ (NEN) nylon membrane. The SA66 probe was made out according to manufacturers' recommendations. The final wash of the membrane was at 65°C in 0.1 X SSPE 0.1% SDS.

Example 3-RT-PCR

RT-PCR analysis was carried out on oilseed rape pod tissu dissected into

DZ and NON-DZ tissue of the pod, embryo, seed coat and funiculus tissu s.

Total RNA that had been extracted by the polysomal method, for which the concentration was known, was diluted to 20ng/μl and 2μl was used for first strand cDNA synthesis as follows. To 20ng mRNA or total RNA 1μl oligo dT (1mg/ml) and 9.6μl milli Q water were added. The eppendorf was vortexed, pulsed in a microcentrifuge and incubated at 70°C for 5 min. After a 5 min cooling period at RT the following were added; 2μl first strand 10X buffer, 2μl 10mM dNTPs (Pharmacia), 2μl 100mM DTT (promega), 10U RNA guard (Pharmacia) and 20U Stratascript reverse transcriptase (Stratagene UK) and incubated at 37°C for 30-45 min. The total volume of this reaction mix was 20μl. A negative control without reverse transcriptase was included to check for the presence of DNA in the RNA stock.

2μl of this first strand DNA was then used in a PCR reaction as follows: The PCR reaction contained a final concentration of 1x PCR buffer, 1.5U Taq polymerase (Promega), 4μg each of two SAC66 specific primers F1 and R6 which bind to the N' terminal region of SAC66, and 2μl first strand cDNA in a total volume of 20μl and was overlaid with mineral oil (Sigma). PCR was carried out in accordance with the following cycles:

1st programme-1 cycle		95°C	5 min
		50°C	3 min
		72°C	3 min
2nd programme-30 cycles		94°C	1 min
		50°C	2 min
		72°C	2 min
3rd programme-1 cycle		94°C	2 min
		50°C	3 min

72 C 15 min

A positive control of first strand DNA generated from 40ng mRNA extracted from the DZ tissue of oilseed rape pods 40, 45 and 50 DAA (as used for the library), first strand cDNA controls without reverse transcriptase and negative PCR controls without DNA were included.

Loading buffer (2μl) was added to the completed PCR reactions and then 10μl was run on a 2% agarose gel. To establish the nature of the band produced during PCR the gel was blotted and probed with a SAC66 cDNA insert.

Total RNA was also extracted from seed coat, funiculus and embryo tissues in addition to combined DZ and NON-Z regions of the pod wall by the small scale RNA method. RNase free DNase (1μl) was added to the RNA in a final concentration of 40mM Tris-HCl pH 7.5, 6mM $MgCl_2$ and final volume of 50μl. The mixture was incubated at 37°C for 10 min and then phenol:chloroform extracted twice. RNA was precipitated with 0.1 volumes 3M NaAc pH 6 and 2.5 volumes 100% ethanol and resuspended in 20μl milliQ water. First strand cDNA synthesis and PCR reactions were carried out as detailed above. However, since it was not possible to quantify the yield of RNA obtained from the small scale preparation it was necessary to amplify the constitutively expressed 25S ribosomal RNA gene in the PCR reactions to ensure that RNA was present and first strand cDNA had been synthesised.

Results

Agarose gel electrophoresis results of the RT-PCR products are shown in figures 5A, 5B and 6. Figures 5A and 5B show that SAC66 mRNA clearly accumulates in the dehiscence zone prior to dehiscence and that no such accumulation occurs in non-zone tissues. In figure 6 it can be seen that SAC66 m ssage is localis d to pod wall valves and funiculus tissue.

Example 4-Ribonuclease Protection Assays

SAC66 plasmid was prepared as described hereinabove. The plasmid was linearized with *Vsp*I which cuts once in the SAC66 sequence at position 1067bp and also restricts at position 2218bp in pBluescript vector sequence to produce a fragment of 2Kb. The restriction digest was run on a 1% agarose gel and DNA from the 2Kb fragment eluted using the Geneclean method. The eluted DNA was ethanol precipitated, washed in 70% ethanol and resuspended in milliQ water to a 5μg/μl concentration.

The 2Kb fragment (1μg) was labelled with 2μl ^{32}P rUTP in a reaction mix containing 1μl DTT (100mM), 2μl 5x buffer (200mM Tris-HCl pH 7.5, 30mM $MgCl_2$, 10mM spermidine, 50mM NaCl), 2μl nucleotide mix containing 2.5 mM each of rCTP, rATP rGTP, 1μl rUTP (100μM), 20U RNase Guard and 1μl T7 RNA polymerase. The T7 RNA polymerase synthesised a 66bp antisense strand to the 3' region of SAC66 from the pBluescript T7 promoter site which was present in the 2Kb fragment. The reaction was incubated at room temperature for 30 min and then 10U RQ1 DNase was added and incubated at 37°C for 15 min to digest the DNA template. Loading dye (10μl of 80% formamide, 10mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, 0.1% SDS) was added and the samples placed in a 65°C heating block for 5 min. The samples were run on a 5% acrylamide protein gel (Longranger) in 0.5x BBEbuffer at 250V.

When electrophoresis was complete the gel was briefly exposed to film (4 min). The autoradiograph was used to determine the position of the full length probe which was excised and eluted overnight at 37°C in 500μl elution buffer (0.5M ammonium acetate, 1mM EDTA and 0.2% SDS).

The following day the supernatant containing the eluted probe was transferred to a fresh tube and diluted with milliQ water until a 20μl aliquot contained approximately 50cps. The probe (20μl) was added to total RNA (10μg) samples and then both the RNA and probe were precipitated with 0.1 volume 3M NaAC and 2.5 volumes ethanol at -20°C

for 20 min.

Digested and undigested controls were included containing a similar quantity of yeast tRNA. The digested control will show the efficiency of RNase One to digest single stranded RNA and the undigested control shows that the probe was added to excess.

After centrifugation at 13,000 rpm for 5 min, ethanol was removed and the pellet allowed to air dry before being resuspended in 30µl hybridization buffer (80% deionized formamide, 40mM PIPES pH 6.4, 0.4M NaAc, 1mM EDTA). The samples were heated to 80°C for 5 min to denature the RNAs and then hybridized overnight at 42°C.

On the subsequent day 30µl of the hybridization mix was added to 300µl RNase digestion buffer (10mM Tris-HCL pH 7.5, 5mM EDTA, 200mM NaAC) containing 2µl RNase One and incubated at 37°C for 2h. To the undigested control only RNase digestion buffer was added. The reaction was terminated by adding 3.3µl SDS (10%). Yeast tRNA (20µg) was added to help precipitation of the RNA with 2.5 volumes ethanol. After centrifugation the resulting pellet was air-dried and resuspended in 6µl loading buffer (80% formamide, 10mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, 0.1% SDS). The samples were heated to 85°C for 5 min before loading onto a pre-warmed sequencing gel. A 5% acrylamide sequencing gel was prepared as deteiled in the Longranger manual. To the final volume of 50ml, 150µl AMPS and 60µl TEMED were added to generate the polymerization of the acrylamide. The gel was run at approximately 50W to maintain the temperature of the plates at 50°C. When electrophoresis was complete the gel was transferred to 3MM paper and dried at 80°C for 50 min. The dried gel was exposed to film for 20h at -70°C.

Results

The results of the Ribonuclease Protection Assay are shown in figure 7.

It can be s en that this confirms that SAC66 transcript accumulat s in the dehisence zone, with no such accumulation occurring in non-zone tissues.

Example 5-Use of SAC66 Promoters

A promoter fragment has been isolated from an *Arabidopsis* genomic library. PG Southern analysis has indicated that this gene is a single copy in *Arabidopsis* and possibly two copies in *B.napus* (figure 4).

To demonstrate that the putative promoter region of SAC66 is capable of driving the expression of a foreign gene in *B.napus*, transcriptional fusions of the promoters can be made to the *E.coli* gene encoding B-glucuronidase (GUS). Fragments of the clones conyaining the putative promoter region are subcloned into pB1101 (Jefferson *et al*, *EMBRO J.*, 6:3901 (1987)). The GUS constructs are then transformed into *B.napus* using standard transformation techniques. Analysis of the transformed plants demonstrates that GUS activity is preferentially found in the pod shatter zone.

This promoter may then be used to drive other genes including antisense nucleic acid to SAC66 itself. The utility of the SAC66 promoter could also be harnessed by expressing gene fusions to barnase, or other genes that disrupt cellular development or otherwise interfere in the function of the shatter zone in pod shatter, in transgenic plants. Use of the barnase gene to cause cell ablation has been described in EP-A-0344029 and WO-A-9211379, particularly at pages 28 and 29 of the latter document. Transcriptional or translational fusion of the promoter fragments and the transfer of these genes into *B.napus* plants results in ablation of the pod shatter zone causing shatter-resistance.

Example 6-The construction of Expression Cassettes and their use in Producing Sense and Antisense RNA to Pod Shatt r Zone-Specific Messages in transgenic Plants

Either pod shatter zone-specific or constitutive promoters can be used to drive expression of sense or anti-sense RNA corresponding to shatter zone-specific transcripts in transgenic plants, thus potentially creating pod mutations and shatter-resistance (indehisence). the same pod shatter zone-specific promoters can be used to drive the pod shatter zone expression of genes encoding proteins or enzymes detrimental to shatter-zone function thereby creating shatter resistance (see previous example). Chimeric genes that can be constructed to produce shatter-resistance include Pea plastocyanin promoter linked to the coding region of the SAC66 cDNA or gene such that sense or antisense SAC66 RNA is produced (see figure 8).

Any other suitable promoter may be used. The plasmids may also carry the npt 11 gene which confers resistance to the antibiotic Kanamycin enabling transgenic plants to be selected on Kanamycin containing media. These plasmids are transformed into *B.napus* using standard Agrobacteriubased nethods as described in the art (Moloney *et al, Plant Cell reports*, 8:238-242 (1989)). Accumulation of expression of the dehisence-zone specific polygalacturonase enzyme results in oilseed rape with pods which are resistant to shattering.

CLAIMS

1. The use of a recombinant or isolated nucleic acid sequence which encodes the enzyme polygalacturonase in the control of dehiscence.

2. The use as claimed in claim 1 wherein the polygalacturonase is preferentially or specifically expressed in the dehiscence zone of pericarp tissue.

3. The use as claimed in claim 2 wherein the pericarp tissue is from the genus *Brassica*.

4. The use as claimed in claim 3 wherein the pericarp tissue is from *Brassica napus*.

5. The use as claimed in any one of claims 1 to 4 wherein the nucleic acid sequence is a DNA sequence.

6. The use as claimed in any one of claims 1 to 5 wherein the nucleic acid sequence is that shown in figure 1, or a sequence substantially homologous thereto.

7. The use as claimed in any one of claims 1 to 5 wherein the nucleic acid sequence includes a promoter or other regulatory sequence which naturally controls expression of polygalacturonase.

8. The use as claimed in any one of claims 1 to 5 wherein the nucleic acid includes a promoter which comprises one or more regulatory elements from a promoter or regulatory sequence which naturally controls expression of polygalacturonase.

9. The use as claimed in any one of claims 1 to 8 wherein the nucleic acid sequence is used to transform a plant or plant propagating material

to produce a transformed plant or plant propagating material.

10. The use of the enzyme polygalacturonase in the control of dehiscence.

11. A nucleic acid sequence which is a promoter or other regulatory sequence which naturally controls expression of polygalacturonase.

12. A nucleic acid sequence as claimed in claim 11 modified by any one or more of the features of any one of claims 2 to 7.

13. The use of a nucleic acid sequence as defined in claim 11 or claim 12 in the control of dehiscence.

14. The use of a recombinant or isolated nucleic acid sequence which is antisense to a nucleic acid sequence as defined in any one of claims 1 to 8 in the control of dehiscence.

15. The use as claimed in claim 14 wherein the antisense nucleic acid sequence includes a promoter or other regulatory sequence.

16. The use as claimed in claim 15 wherein the promoter is a constitutive promoter or is a promoter which naturally controls expression of polygalacturonase.

17. A transgenic plant which includes a nucleic acid sequence as defined in any one of claims 1 to 8 or claims 10 to 16.

18. An oilseed rape plant which is shatter resistant.

19. An oilseed rape plant as claimed in claim 18 which includes a nucleic acid sequence as defined in any one of claims 1 to 8.

20. A plant cell which includes a nuceic acid sequ nce as defined in any one of claims 1 to 8 or claims 10 to 16.

21. Propagating material derived from a plant as claimed in any one of claims 17 to 19.

22. Seeds derived from a plant as defined in any one of claims 17 to 19.

23. A method of regulating dehiscence, which comprises the step of transforming or transfecting propagating material from a plant with a nucleic acid sequence as defined in any one of claims 1 to 8 or claims 10 to 16.

24. A method as claimed in claim 23 for reducing or preventing dehiscence wherein the nucleic acid sequence is as defined in any one of claims 14 to 16.

FIG.1.

```
GGCATCACGAGGGTACCCGTAAATCCCACCATACAACAAAGTTCTGTGAAAGTCTCCCAA 60

AAACTGCAAAGAGTCTCATATTAGTTCTTACTCTCAGAAATAAAACACACTCTTTCTGAA 120

AAGATTAGCGTTTCAAACCCCGAAATGGCCCGTTGTCATGGAAGTCTTGCTATTTTCTTA 180
                          M  A  R  C  H  G  S  L  A  I  F  L  12
TGCGTTCTTTTGATGCTCGCTTGCTGCCAAGCTTTGAGTAGCAACGTAGATGATGGATAT 240
 C  V  L  L  M  L  A  C  C  Q  A  L  S  S  N  V  D  D  G  Y  32
GGTCATGAAGATGGAAGCTTCGAAACCGATAGTTTAATCAAGCTCAACAACGACGACGAC 300
 G  H  E  D  G  S  F  E  T  D  S  L  I  K  L  N  N  D  D  D  52
GTTCTTACCTTGAAAAGCTCCGATAGACCCACTACCGAATCATCAACTGTTAGTGTTTCG 360
 V  L  T  L  K  S  S  D  R  P  T  T  E  S  S  T  V  S  V  S  72
AACTTCGGAGCAAAAGGTGATGGAAAAACCGATGATACTCAGGCTTTCAAGAAAGCATGG 420
 N  F  G  A  K  G  D  G  K  T  D  D  T  Q  A  F  K  K  A  W  92
AAGAAGGCATGTTCAACAAATGGAGTGACTACTTTCTTGATTCCTAAAGGGAAGACTTAT 480
 K  K  A  C  S  T  N  G  V  T  T  F  L  I  P  K  G  K  T  Y  112
CTCCTTAAGTCTATTAGATTCAGAGGCCCATGCAAATCATTACGTAGCTTCCAGATCCTA 540
 L  L  K  S  I  R  F  R  G  P  C  K  S  L  R  S  F  Q  I  L  132
GGCACTTTATCAGCTTCTACAAAACGATCGGATTACAGTAATGACAAGAACCACTGGCTT 600
 G  T  L  S  A  S  T  K  R  S  D  Y  S  N  D  K  N  H  W  L  152
ATTTTGGAGGACGTTAATAATCTATCAATCGATGGCGGCTCGGCGGGGATTGTTGATGGC 660
 I  L  E  D  V  N  N  L  S  I  D  G  G  S  A  G  I  V  D  G  172
AACGGAAAAATCTGGTGGCAAAACTCATGCAAAATCGACAAATCTAAGCCATGCACAAAA 720
 N  G  K  I  W  W  Q  N  S  C  K  I  D  K  S  K  P  C  T  K  192
GCGCCAACGGCTCTTACTCTCTACAACCTAAACAATTTGAATGTGAAGAATCTGAGAGTG 780
 A  P  T  A  L  T  L  Y  N  L  N  N  L  N  V  K  N  L  R  V  212
AGAAATGCACAGCAGATTCAGATTTCGATTGAGAAATGCAACAGTGTTGATGTTAAGAAT 840
 R  N  A  Q  Q  I  Q  I  S  I  E  K  C  N  S  V  D  V  K  N  232
GTTAAGATCACTGCTCCTGGCGATAGTCCCAACACGGATGGTATTCATATCGTTGCTACT 900
 V  K  I  T  A  P  G  D  S  P  N  T  D  G  I  H  I  V  A  T  252
AAAAACATTCGAATCTCCAATTCAGACATTGGGACAGGTGATGATTGCATATCCATTGAG 960
 K  N  I  R  I  S  N  S  D  I  G  T  G  D  D  C  I  S  I  E  272
GATGGATCGCAAAATGTTCAAATCAATGATTTAACTTGCGGCCCCGGTCATGGCATCAGC 1020
 D  G  S  Q  N  V  Q  I  N  D  L  T  C  G  P  G  H  G  I  S  292
ATTGGAAGCTTGGGGGATGACAATTCCAAAGCTTATGTATCGGGAATTAATGTGGATGGT 1080
 I  G  S  L  G  D  D  N  S  K  A  Y  V  S  G  I  N  V  D  G  312
GCTACGCTCTCTGAGACTGACAATGGAGTAAGAATCAAGACTTACCAGGGAGGGTCAGGA 1140
 A  T  L  S  E  T  D  N  G  V  R  I  K  T  Y  Q  G  G  S  G  332
ACTGCTAAGAACATTAAATTCCAAAACATTCGTATGGATAATGTCAAGAATCCGATCATA 1200
 T  A  K  N  I  K  F  Q  N  I  R  M  D  N  V  K  N  P  I  I  352
ATCGACCAGAACTACTGCGACAAGGACAAATGCGAACAACAAGAATCTGCGGTTCAAGTG 1260
 I  D  Q  N  Y  C  D  K  D  K  C  E  Q  Q  E  S  A  V  Q  V  372
AACAATGTCGTGTATCGGAACATACAAGGTACGAGCGCAACGGATGTGGCGATAATGTTT 1320
 N  N  V  V  Y  R  N  I  Q  G  T  S  A  T  D  V  A  I  M  F  392
AATTGCAGTGTGAAATATCCATGCCAAGGTATTGTGCTTGAGAATGTGAACATCAAAGGA 1380
 N  C  S  V  K  Y  P  C  Q  G  I  V  L  E  N  V  N  I  K  G  412
GGAAAAGCTTCTTGCAAAAATGTCAATGTTAAGGATAAAGGCACCGTTTCTCCTAAATGC 1440
 G  K  A  S  C  K  N  V  N  V  K  D  K  G  T  V  S  P  K  C  432
CCTTAATTACTAAGTTGATTATGTAATATACATAAATACGTATTATATGTGGTTATAGAT 1500
 P                                                             433
GCCATCTATATCCTTATCTACGTATTGATTCTCGATATATATAGAAAACTAAGGATTTAT 1560

GGGAATATACATACAATAGTTGAGATAATTGTTGTCTTGTATATGGTTCACTGAAGTTGA 1620

TTGCTTGTCCACGAATAAATGAATAATGTCATTTGTC                         1657
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FIG. 2.

```
TGGCGAATTCCGAATACGGACGGTATTCATATCGTTGCTACTAAA 45
      P  N  T  D  G  I  H  I  V  A  T  K  12

AACATTCGAATCTCCAATTCAGACATTGGGACAGGTGATGATTGC 90
 N  I  R  I  S  N  S  D  I  G  T  G  D  D  C  27

ATATCCATTGAGGATGGATCGCAAAATGTTCAAATCAATGATTTA 135
 I  S  I  E  D  G  S  Q  N  V  Q  I  N  D  L  42

ACTTGCGGCCCCGGTCACGGCCTAGGTGG                 164
 T  C  G  P  G  H  G                          49
```

FIG. 3.

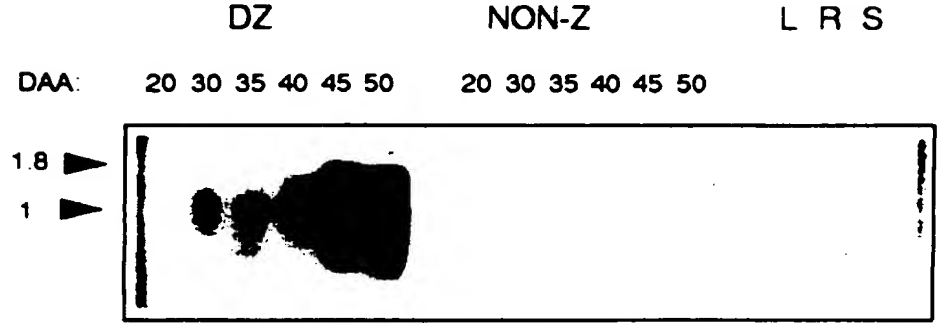
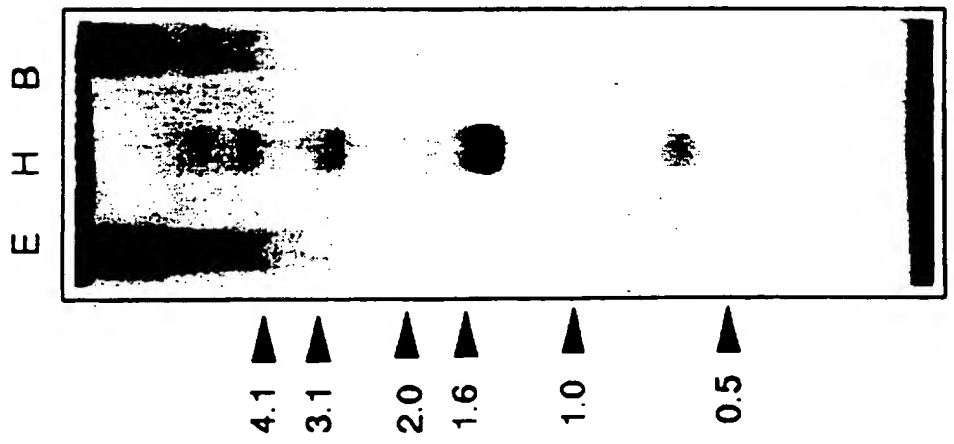
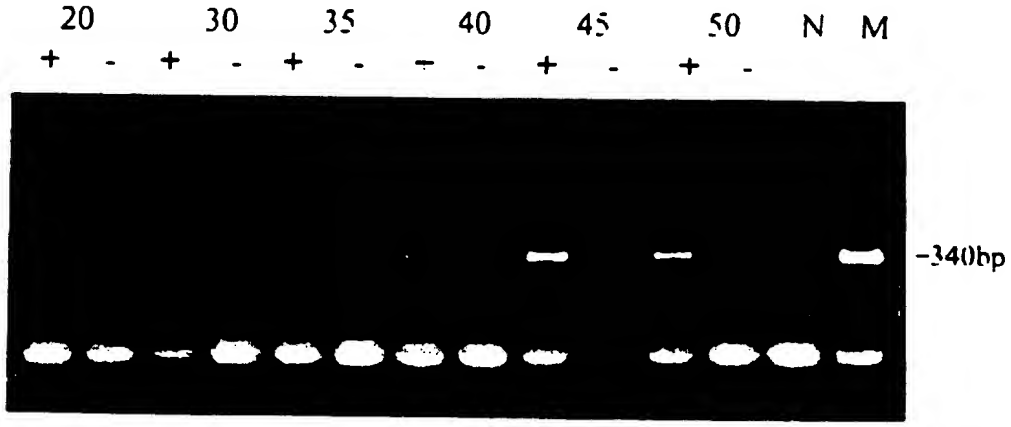


FIG. 4.



DZ

FIG. 5A.



NON-Z

FIG. 5B.

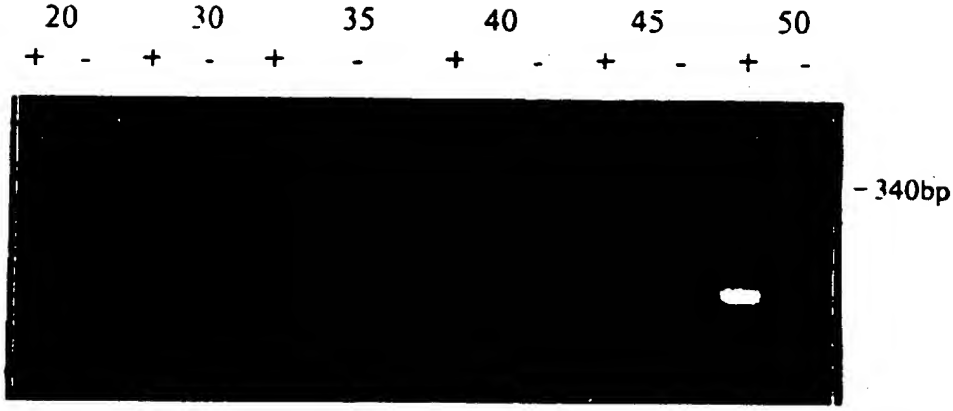


FIG. 6.

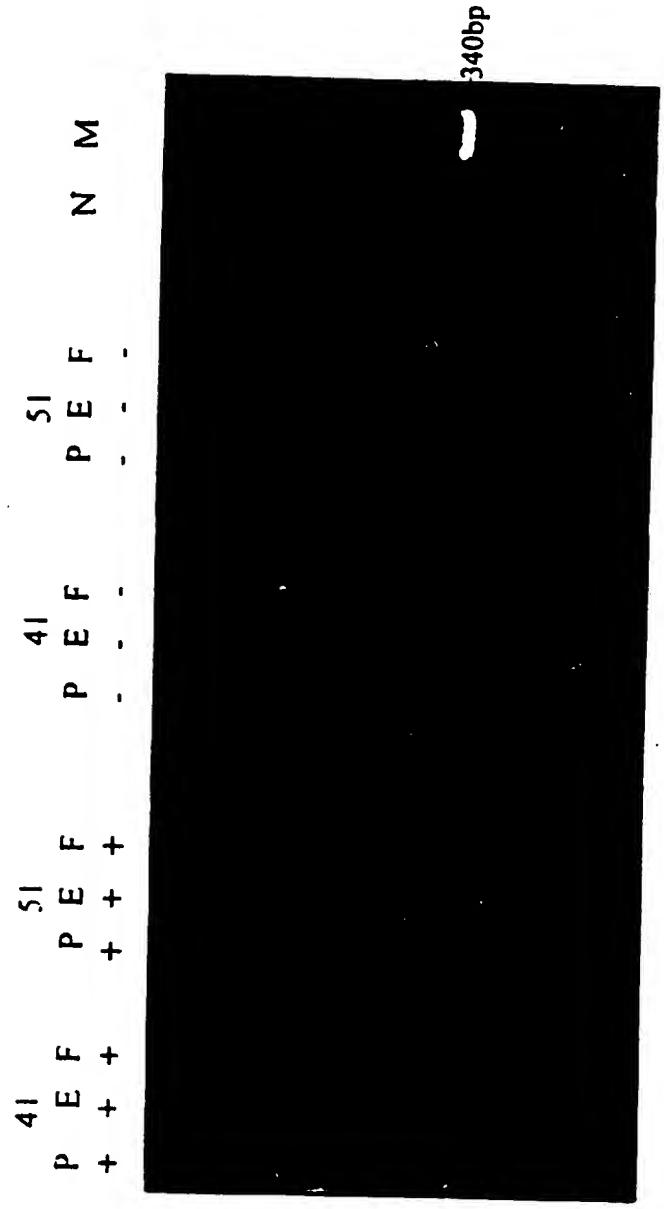


FIG. 7.

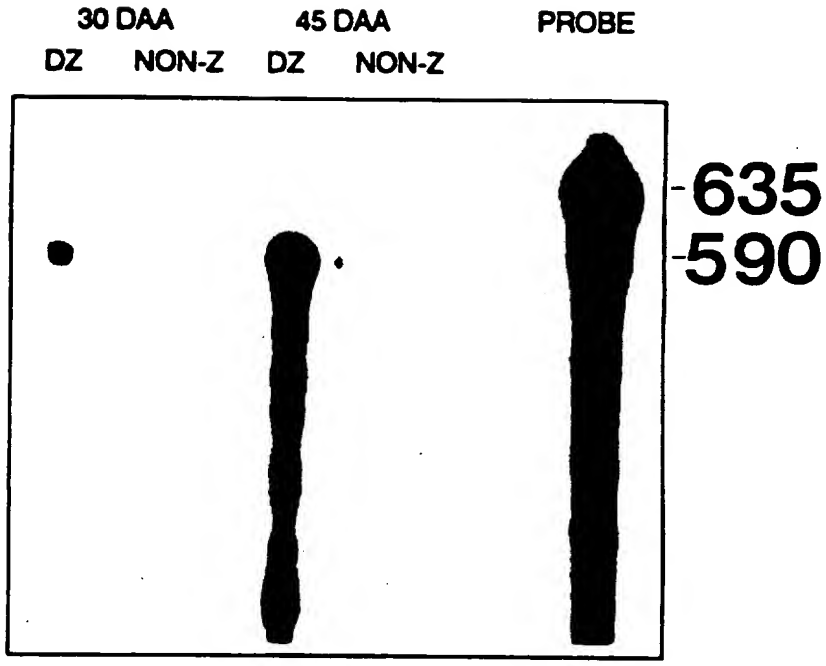
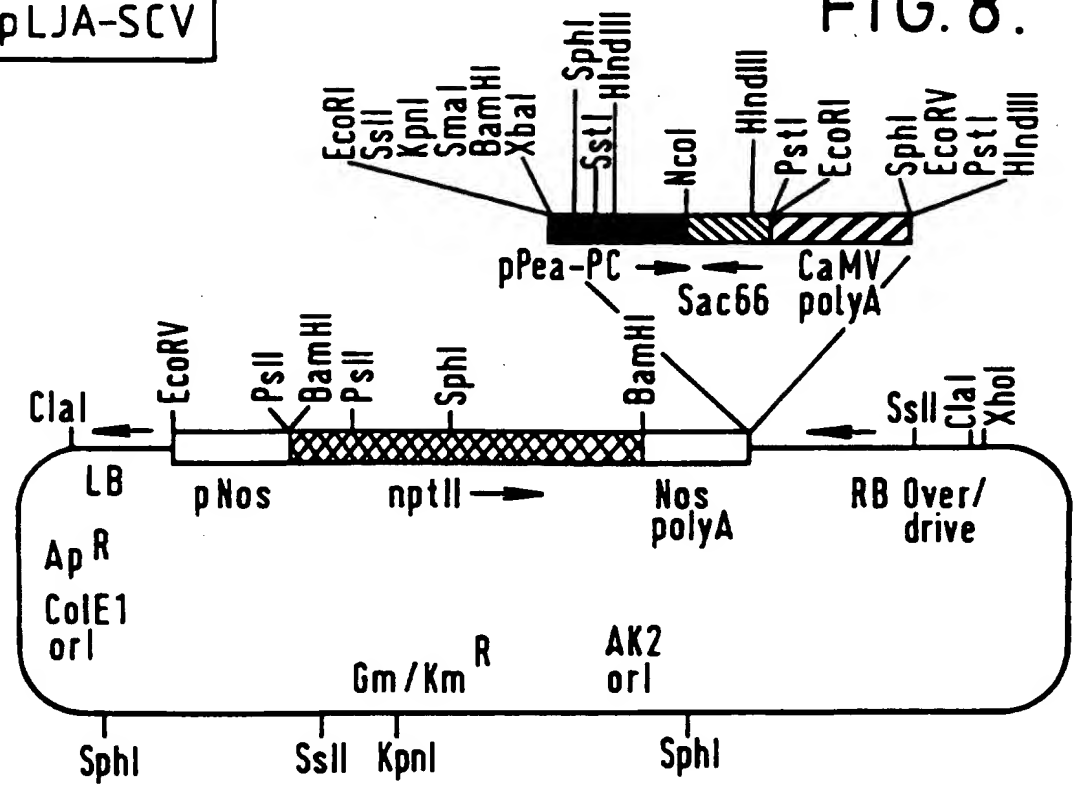
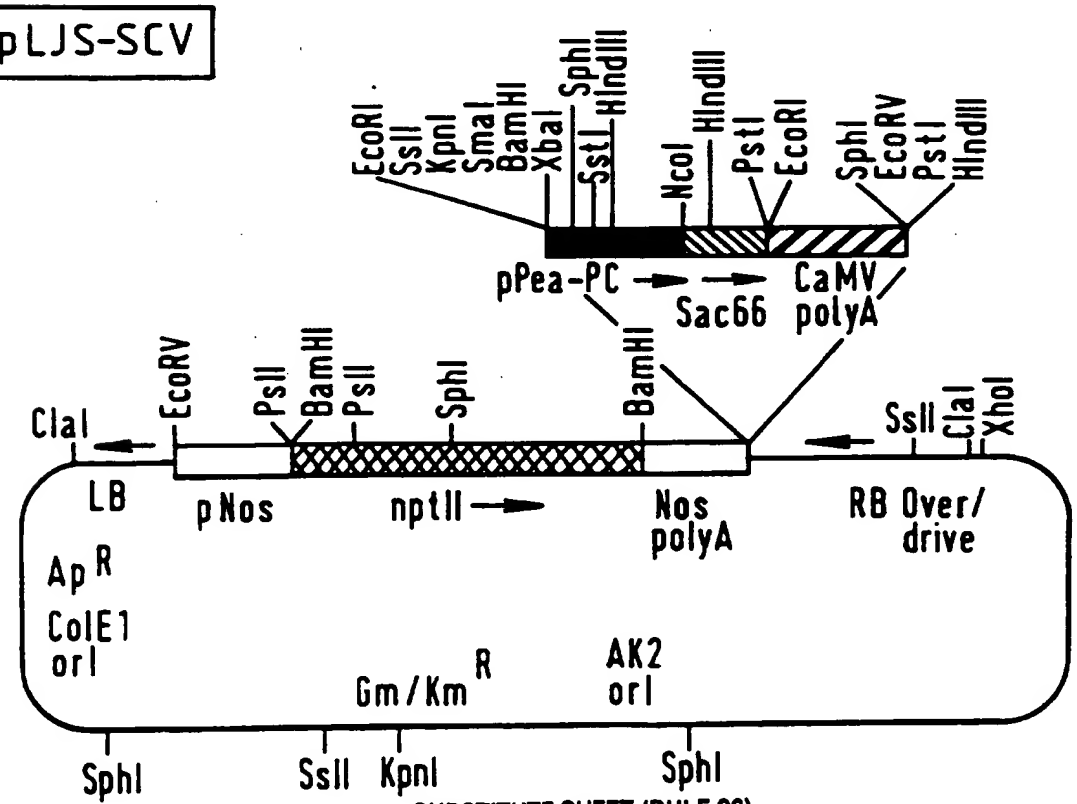


FIG. 8.

pLJA-SCV



pLJS-SCV



INTERNATIONAL SEARCH REPORT

Ir. tional Application No
PCT/GB 96/00757

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/56 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 01572 (PIONEER HI BRED INT) 20 January 1994 see whole document particularly page 20 lines 1-15 ---	11
X	PLANT MOLECULAR BIOLOGY, vol. 11, 25 August 1988, pages 651-662, XP002002156 BIRD C R ET AL: "THE TOMATO POLYGALACTURONASE GENE AND RIPENING-SPECIFIC EXPRESSION IN TRANSGENIC PLANTS" see the whole document --- -/--	11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
27 August 1996	03.09.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Maddox, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GENET RES 56 (1). 1990. 1-2. , XP000574919 PRAKASH S ET AL: "RECONSTRUCTION OF ALLOPOLYPLOID BRASSICAS THROUGH NONHOMOLOGOUS RECOMBINATION INTROGRESSION OF RESISTANCE TO POD SHATTER IN BRASSICA - NAPUS." see the whole document	18-24
X	WO,A,94 23043 (NICKERSON BIOCEM LTD ;ISAAC PETER GEOFFREY (GB); ROBERTS JEREMY AL) 13 October 1994 see the whole document	18-24
P,X	ANNUAL MEETING OF THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, CHARLOTTE, NORTH CAROLINA, USA, JULY 29-AUGUST 2, 1995. PLANT PHYSIOLOGY (ROCKVILLE) 108 (2 SUPPL.). 1995. 75., XP002002155 PETERSEN M., ET AL.: "An endo-polygalacturonase isoform potentially involved in pod dehiscence in oilseed rape ." * abstract 342 *	1-10, 17-24
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